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Role of Endothelial Nitric Oxide Synthase (T-786C) Gene Polymorphism in the Development of Coronary Artery Disease

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ABSTRACT

Background: Recent studies suggest a cause and effect relationship between eNOS gene polymorphism and increase incidence of insulin resistance as NO deficiency is associated with decreased vasodilation and altered binding of insulin to insulin receptor.
Aim: This study aimed to study eNOS -786T/C gene polymorphism in patients with CAD in Indian population.
Methodology: This study consisted of 60 adult patients of with documented CAD and 60 age and sex matched healthy subjects as controls. Fasting Serum Insulin was measured by ELISA, fasting Serum nitric oxide by modified Griess reaction and fasting plasma glucose on fully automated chemistry analyzer (Hitachi 902) HOMA-IR was calculated mathematically by using formula given by Matthew et al. The eNOS gene loci was amplified by using PCR and by RFLP. A p-value <0.05 was considered significant. Statistical analysis was performed with the help of SPSS version.
Results: The mean serum NO levels were very significantly lower in case groups and mean HOMA-IR levels in the study group were very significantly higher as compared to the control group (p=0.000) suggesting the role of insulin resistance in CAD. The frequency of T allele was 76.67% in the study group and 81.67% in the control group while the frequency of C allele was 23.33% in the study group 18.33% in the control group respectively. This difference was found to be statistically significant. (p=0.0123)
Conclusions: Endothelial Nitric Oxide Synthase (T-786C) gene polymorphism is a significant risk factor for development of CAD.

Key words: Endothelial nitric oxide synthase (T-786C), gene polymorphism, Coronary artery disease

INTRODUCTION

During the last decade, a multitude of experimental arguments have led to the concept that NO is not only involved in the control of vasomotor tone but also in vascular homeostasis and neuronal and immunological functions. Recent studies suggest a cause and effect relationship between eNOS gene polymorphism and increase incidence of insulin resistance as NO deficiency is associated with decreased vasodilation and altered binding of insulin to insulin receptor[1-6]. However, several other studies have shown variable results. Current studies worldwide suggest an association between eNOS -786T/C gene polymorphism and genetic susceptibility to insulin resistance. Studies suggest eNOS-786T/C gene polymorphism is distinct in specific population group, ethnicity and geographic region and perhaps this genetic variability might produce different results on exposure to various environmental factors. Besides there is hardly any data available in Indian population.[7,8]So further research is required to explore the complex interaction between environmental factors and eNOS -786T/C gene polymorphism in susceptibility to insulin resistance in patients with CAD in Indian population[9-12].

AIMS AND OBJECTIVES

This study aimed to study endothelial Nitric Oxide Synthase (T786C) gene polymorphism, Nitric oxide level and

Insulin Resistance in patients with Coronary Artery Disease

METHODOLOGY

The study was conducted in Department of Biochemistry and Department of Cardiology, Vardhman Mahavir Medical College and Safdarjung hospital, New Delhi.

This was a hospital based case – control (observational) study conducted on patients attending Cardiac OPD in Safdarjung Hospital, New Delhi.

The study population consisted of 60 adult patients of either sex with documented CAD. Consisted of 60 age and sex matched healthy subjects.

INCLUSION CRITERIA:

Angiographically proven cases of coronary artery disease were included in the study.

EXCLUSION CRITERIA:

1. Diagnosed cases of Type 1 & 2 Diabetes mellitus.
2. Patients with congenital heart diseases.
3. Chronic kidney and liver disease.
4. Any history of debilitating illness. Any history of drugs affecting NO levels.

The study was conducted after institutional Ethical Committee approval and informed consent was taken from all patients and controls.

Bilingual informed written consent was taken from the patients. Detailed clinical history with special reference to Coronary Artery Disease and thorough clinical examination of patient was conducted. Necessary anthropometric measurements like height, weight and body mass index were taken. Venous blood was collected from subjects under sterile conditions after overnight fasting. The whole blood collected in EDTA vacutainer was transferred to eppendorf and stored at -20 degree Celsius till DNA was extracted for PCR and RFLP. Nitric oxide in serum was determined indirectly by the measurement of its stable decomposition product nitrite (NO_2), by employing the Griess reaction according to the modified method of Mathew et al[13].

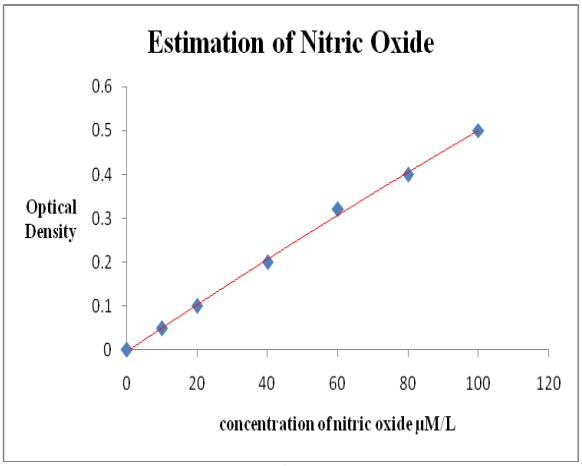


FIGURE 1 : Standard curve of Nitric oxide obtained at 543nm

Total nitrite was determined from the standard slope constructed from known standard concentration and their corresponding absorbance values.

Fasting Serum Insulin Estimation: The estimation of serum Insulin was performed by sandwich ELISA technique. The kit was procured from Calbiotech Inc., CA.

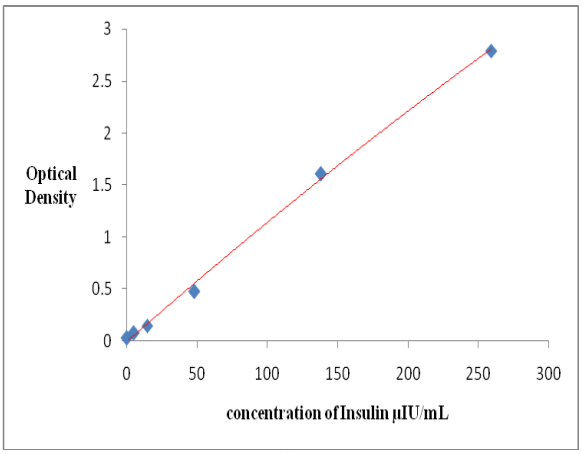


FIGURE 2: Standard curve of serum insulin obtained at 450 nm

Estimation of Blood Glucose

Blood Glucose estimation was done by Glucose oxidase peroxidase method using commercially available kit Randox GL 7952 on automated chemistry analyser Hitachi 902.

HOMA-IR (Homeostasis Model of Assessment- Insulin Resistance):

Insulin resistance was calculated mathematically by using formula given by Matthew et al[14].

fasting Glucose(mg/dl) x fasting Insulin(µU/mL) / 405

Manual DNA extraction by the method of Daly's et al [15].

POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION:

The required region of NOS3 gene from genomic DNA was amplified by Polymerase Chain Reaction in MJ Research PTC-100^{TM}(Peltier Thermal Cycler).

PRIMER SEQUENCE FOR NOS3

FORWARD PRIMER:- 5'-GTCTCTCAGCTTCCGTTTCTT-3'

REVERSE PRIMER:- 5'-CCTTGAGTCTGACATTAGGGTATC-3'

.These primers were used to amplify a 458bp product for T-786C (rs2070744)

REAGENTS REQUIRED

1. Template DNA (e.g. genomic DNA): 200-300ng
2. Forward and reverse PCR primers (Active Oligos): 0.3pM
3. $MgCl_2$ (present in PCR Buffer) :20 mM
4. dNTPs (a mixture ofdATP, dCTP, dGTP, anddTTP): (Thermo Fischer Scientific) 200µM
5. 10× PCR buffer (Thermo Fischer Scientific): 1X
6. TaqDNA polymerase (Dream Taq,Thermo Fischer Scientific): 0 .3pM
7. Water to make total volume up to 20 µl.

The thermal cycling conditions were carried out in a PTC-100^{TM} (Peltier Thermal Cycler) machine as follows-

Denaturation at 95^{0}C for 2 min,30 cycles of denaturation at 95^{0}C for 30 sec, annealing at 58^{0}C for 30 sec, elongation at 72^{0}C for 90 sec, followed by a final elongation step at 72^{0}C for 10 min.

The PCR products were analysed in a 2% agarose gel in a 1X TAE buffer system. This product was digested with restriction enzyme separately to reveal the genotype for the SNP.

The condition for digestion was as follows:

The PCR product (10 µl) was digested individually with 1µl (10unit/µl) of Msp1 (Krishgen) with 2µl of 10x restriction buffer and 18µl of nuclease free water incubated at 37^{o}C for 16 hours. Digested product was analysed in a 2% agarosegel.The TT genotype produced two fragments of 303bp and 155bp, while TC genotype produced three fragments of 257bp, 155bp and 46bp.

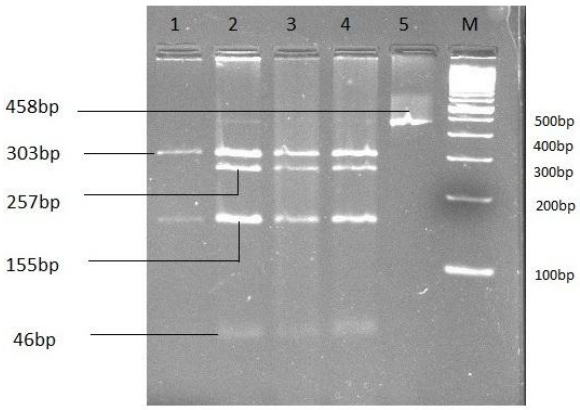


FIGURE 3: Ethidium bromide-stained agarose gel used for genotyping PCR product digested with Msp1.Lane1: Homozygote (TT) showing two bands at 303bp and155bp.
Lane 2,3& 4: Heterozygotes (TC) showing four bands at 303bp, 257bp, 155bp and 46bp.Lane M: molecular wt marker

STATISTICAL ANALYSIS:

A p-value <0.05 was considered significant. Statistical analysis was performed with the help of SPSS version 20. The data was subjected to t-test & dichotomous variables and allele frequencies were analyzed by Chi-Square test. For correlation of two continuous variables, correlation coefficient was used.

RESULTS

DEMOGRAPHIC DISTRIBUTION

The study population consisted of 56.66% males and 43.33% females whereas the control group consisted of 60% males and 40% females.

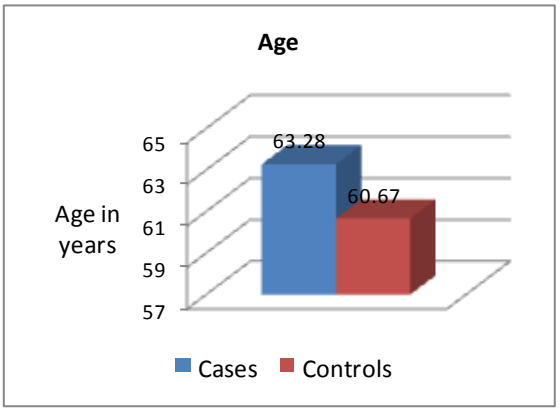


FIGURE 4: AGE DISTRIBUTION OF CASES AND CONTROLS

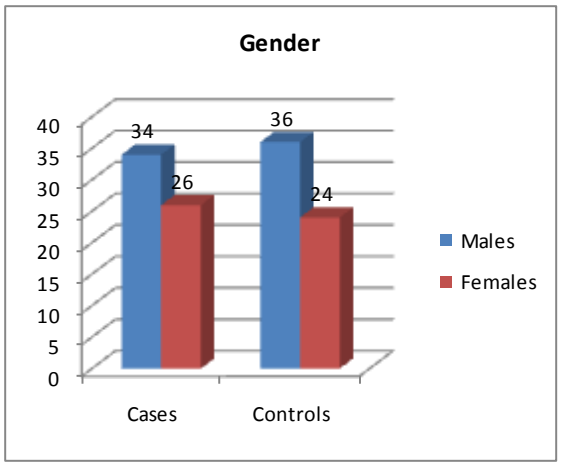


FIGURE 5: GENDER DISTRIBUTION OF CASES AND CONTROLS

TABLE 1: DEMOGRAPHIC AND ANTHROPOMETRIC PARAMETERS OF STUDY GROUP

Parameters	Cases (n=60) (Mean±SEM)	Controls (n=60) (Mean±SEM)	p value
Height(metres)	1.641±0.01	1.649±0.01	0.612
Weight(kg)	70.20±1.662	67.73±1.583	0.285
Bmi(kg/metre^2)	25.89±0.40	24.83±0.45	0.085
Systolic BP(mm of Hg)	131.93±0.91	126.16±1.03	0.000*
Diastolic BP(mm of Hg)	80.57±0.83	80.27±0.76	0.791

*p≤0.000 is statistically very significant

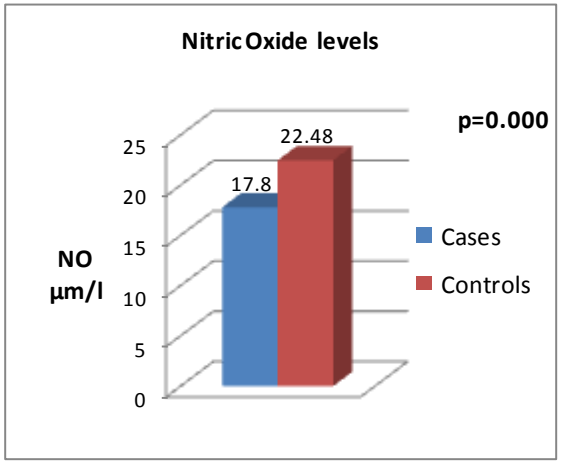
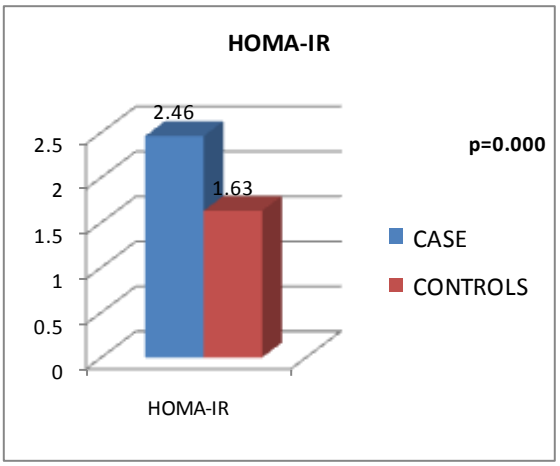


FIGURE 6: NITRIC OXIDE LEVELS IN CASES AND CONTROLS

TABLE 2: GLYCEMIC PROFILE OF STUDY POPULATION

Parameters	Cases (n=60) (Mean±SD)	Controls (n=60) (Mean±SD)	p value
FPG	95.33±2.92	93.06±3.95	0.001*
Insulin	10.41±4.72	7.10±2.94	0.000*
HOMA-IR	2.46±1.16	1.63±0.69	0.000*

*p≤0.000 is statistically very significant



*p≤0.000 is statistically very significant

FIGURE 7: HOMA-IR IN CASES AND CONTROLS

The mean HOMA-IR was higher in the cases (2.46±1.16) as compared to the controls(1.63±0.69) , with statistically significant difference (p=0.000).

TABLE 3: DISTRIBUTION OF GENOTYPE OF T-786C (rs2070744) POLYMORPHISM IN STUDY AND CONTROL GROUP

Genotype	Cases (n=60)		Controls (n=60)		Level of significance
	N	Frequency	N	Frequency	
TT	32	53.33	38	63.33	0.0123
TC	28	46.66	22	36.66	
CC	0	0	0	0	

Endothelial Nitric Oxide (eNOS) gene variants (T786-C) was determined in both groups. TT genotype was found in 32 subjects in the study group (53.33%) and in 38 subjects in the control group (63.33%), whereas the TC genotype was found in 28 subjects in the study group (46.66%) and in 22 subjects in the control group (36.66%) respectively. No CC genotype was found in any group. The genotype distribution was in Hardy Weinberg equilibrium. The frequency of T allele was 76.67% in the study group and 81.67% in the control group while the frequency of C allele was 23.33% in the study group 18.33% in the control group respectively. This difference was found to be statistically significant. (p=0.0123, table 3)

DISCUSSION

The effect of endothelial nitric oxide synthase gene polymorphism on Insulin resistance has not been widely reported until

now. In the present study we have evaluated the effect of endothelial nitric oxide synthase gene polymorphism (assessed by RFLP and nitric oxide levels) on insulin resistance (calculated by HOMA-IR) in patients of Coronary Artery Disease.[16-18]

In our study we found that the mean systolic blood pressure was higher in the cases 131.93±0.91 mm of Hg as compared to the controls 126.16±1.03 mm of Hg, with statistically very significant difference (p=0.000). Our findings are similar to the study done by Garg and colleagues [19-21]. The probable reason for high BP in these patients is insulin resistance and the resultant hyperinsulinemia which increases BP by activation of the sympathetic nervous system and rennin-angiotensin-aldosterone system (RAAS) resulting in sodium retention and volume expansion[22]. Activation of RAAS produces angiotensin II which acts through angiotensin I receptors, thus inhibits the vasodilatory effects of insulin on blood vessels and increasing BP. Hyperinsulinemia in insulin resistant state also stimulates the mitogen-activated protein kinase (MAPK) pathway, which promotes vascular injury.

NO can be seen in most tissues and cells and plays an essential role in regulating vascular tone and hemodynamic. NO stimulates endothelial proliferation and angiogenesis, thereby playing an important role in microcirculation. In addition, NO inhibits the release of endothelin-1 (a vasoconstrictor). Its most prominent roles in cardiovascular system are blood pressure regulation, inhibition of thrombocyte aggregation, leukocyte adhesion, smooth muscle cell proliferation, and LDL oxidation. The mean plasma nitric oxide level in the study group cases was 17.80±0.95 µM/L and in the control group was 22.47±0.83 µM/L. The difference between the two was statistically significant (p=0.000). The decrease in production in patients are associated with events that accelerate development of atherosclerosis such as vasoconstriction, thrombocyte aggregation, migration of monocytes to the vascular wall, oxidized LDL and foam cell production.[23]

Our study is in accordance with studies by Flammeret al[24] and Luscher et al[25], who have postulated that Low levels of NO in patients is suggestive that patients are more likely to have accelerated development of atherosclerosis.

In our study we found that the markers of insulin resistance were significantly raised in cases as compared to controls. The mean HOMA-IR levels in the study group were 2.46±0.15 and in the control group was 1.63±0.09. The difference between the two groups was very significant (p=0.000). The mean serum insulin levels in the study group were 10.41±0.61µIU/mL and in the control group were 7.10±0.38µIU/mL and the difference between the two groups was statistically very significant (p=0.000).

Bertoluci et al[26] suggested that increased HOMA-IR is positively associated with angiographic Coronary Artery Disease and may be useful for risk stratification as a high specificity test for Coronary Artery Disease. The probable reason for increased cardiovascular risk in patients with insulin resistance is that insulin resistance in adipocytes leads to reduced uptake of circulating lipids and increased hydrolysis of stored triglycerides.[27] Increased mobilization of stored lipids from these cells would elevate free fatty acids in blood thereby causing dyslipidemia, which would predispose the patient to increased cardiovascular risk. Other studies have also proposed that insulin resistance accentuates the risk of CAD. Our findings suggest that TT genotype may have a protective role in development of insulin resistance and coronary artery disease as seen by increased frequency of TT genotypes than TC genotypes in case as compared to control groups. These findings are similar to findings of previous studies. However, these findings need to be confirmed in a larger sample size.

CONCLUSIONS

It was found that reduced plasma level of Nitric oxide is associated with increased risk of CAD. Also, higher HOMA-IR levels are found in patients with CAD. Moreover, endothelial Nitric Oxide Synthase (T786C) gene polymorphism is a significant risk factor for development of CAD.

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